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A Laminar-Flow Microfluidic Device for Quantitative Analysis of Microbial Electrochemical Activity

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We report a laminar flow-based microfluidic bioelectrochemical system (BES) that was fabricated by using polydimethyl siloxane (PDMS) channels and gold electrodes. The microfluidic BES was operated as a potentiostatically controlled two-electrode system. A pure culture of *Geobacter sulfurreducens* strain PCA, which is a model electrode-respiring bacterium, was grown in the channel and respired with the electrode under strict anaerobic conditions. We took advantage of the short hydraulic retention time ($\approx$2 min) and response times ($<$21 min) to rapidly test the effect of certain chemical stimuli, such as O_2 and anthraquinone disulfide (AQDS), on electric current production by *G. sulfurreducens*. The results showed that: i) short-term (2 min) exposure to O_2-saturated solution did not cause any irreversible toxicity to *G. sulfurreducens*, and ii) AQDS can be used as a redox mediator by *G. sulfurreducens* for shuttling electrons between the microbe and the electrode. We, therefore, demonstrate that the microfluidic BES is a promising research tool for gaining insight into microbial electrochemical activity. In our two-dimensional microfluidic-based research tool, a well-defined electrochemical environment can be maintained with the help of laminar flow without a membrane to separate two electrodes.

Introduction

Bioelectrochemical systems (BESs) harbor electrochemically active bacteria at the electrodes as biocatalysts for redox reactions. BESs can be classified into: i) potentiostatically controlled microbial three-electrode cells (M3Cs), ii) microbial electrolysis cells (MECs), and iii) microbial fuel cells (MFCs). M3Cs are valuable research tools, as a well-defined electrochemical environment is maintained in these devices by poising the working electrode at a constant potential with respect to a reference electrode with the help of a third electrode—the counter electrode. This permits focused investigation into microbial ecology,[1] biofilm formation,[2] and application as versatile biocomputing platforms.[3] By using the same electrode as the counter electrode and reference electrode, these systems can also be condensed into potentiostatically controlled microbial two-electrode systems. This is similar to the second type of BESs—MECs—and they are often used to generate chemical products at the cathode by applying an artificial potential difference between the two electrodes. The third type of BESs—MFCs—are used to generate electric current from the microbially induced potential difference between the anode and the cathode. As both MECs and MFCs utilize the reducing power generated by microbial oxidation of organic substrates at the anode, they have been hailed as a sustainable technology for wastewater treatment,[4] greenhouse gas reduction,[5] and chemical production.[6]

Although considerable BES research has focused on scaling up the reactor size of MECs and MFCs for industrial applications, there is also an increasing interest in miniaturizing BESs as research tools. Miniaturizing BESs to the microliter scale opens up the possibility of new applications. Non-BES microfluidic devices with similar dimensions have been used as versatile investigative tools for bacterial separation,[7] growth condition optimization,[8] cell patterning,[9] bacterial antibiotic resistance,[10] and single-cell-behavior and genetic characterization.[11] However, the use of microfluidic devices to study electrochemically active bacteria is still in its infancy. Miniaturized BESs have been used, thus far, only to perform high throughput screening for electrochemically active bacteria from environmental samples.[12] One of the main difficulties involved in the fabrication of microfluidic BESs is separating the anode and cathode at such small length scales that any cross reactions between the two electrolytes is prevented. In most of the reported microfluidic BESs, thus far, ion exchange membranes have been used to physically separate the anolyte and catholyte, which increases the internal resistance, the fabrication complexity and cost, and neglects the laminar flow of liquids that naturally occurs in such devices.[13] Laminar flow, which refers to fluid flow at Reynolds number (Re) less than 2100, is an important characteristic of microfluidic devices. This allows parallel flow of two separate streams without convective mixing and has been successfully used to separate the anode and cathode in chemical and enzymatic fuel cells.[14]

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Herein, we present a membrane-less microfluidic BES that exploits laminar flow. We used this as a tool to analyze the electrochemical activity of *Geobacter sulfurreducens* in response to different chemicals. This microfluidic BES-based quantitative analysis tool has short hydraulic retention times (HRT, ≈2 min) and fast response times (<21 min), which are much smaller than the doubling time of *G. sulfurreducens* (≈6 h).[15] This provides us with the possibility to use microchannels and the inherent hydrodynamic features to study the immediate effects of different chemical stressors on the microbial electrochemical activity of *G. sulfurreducens* cells. As *G. sulfurreducens* is an obligate anaerobe, strict anaerobic conditions were maintained during operation. Our work shows that this microfluidic device can be a multipurpose tool for BES research.

Results and Discussion

Device startup

Laminar flow is the basis for successful operation of our device as a BES because it is responsible for separating the anolyte and catholyte when diffusion is limited. Based on the combination of the physical dimensions of the channel and the low flow rate of the anolyte and catholyte, we expect a Reynolds number in the range 0.22–1.1, which is well within the range to guarantee laminar flow. We confirmed laminar-flow operating conditions by visualizing the flow pattern in our device with two differently colored liquids (red and green; Figure 1 a). There is no convective mixing between the two fluids implying that the interfacial mixing is principally diffusion driven.

After confirming laminar flow in the channel, we operated our device as a two-electrode potentiostatically controlled system (MEC) with the working electrode poised at 0.7 V versus the counter/reference electrode. The current, which is a direct indicator of bacterial growth and biofilm formation for *G. sulfurreducens*, started increasing in 2–3 days and reached a maximum value of 4–10 μA (100–249.2 μA cm^{-2}) in approximately 10 days (Figure 1 b), after which it remained stable for approximately 22 days. Sustained long-term performance is necessary for using microfluidic BESs as a portable power supply or as a research tool. Previous microfluidic BESs have typically only been operated for several days to a maximum of two weeks.[13a,b,16] To our knowledge, this is the first reported microfluidic BES that has been successfully operated for over one month. The importance of laminar flow for this microfluidic BES-based research tool are: i) in the potentiostatic mode of operation, H_2O is constantly reduced to H_2 and OH^- ions at the cathode, resulting in an increase in the pH value. However, due to laminar flow-based fluid separation in our device, this alkalinization is localized to the catholyte and not responsible for bacterial inhibition, as *G. sulfurreducens* grows solely at the anode; ii) following the chemical-stimuli-injection stage, laminar flow can localize all the chemical injections at the working electrode, and thus eliminate any influence to the catholyte redox status. Due to a constant potential difference artificially applied between working electrode and counter/reference electrode, a stable redox status at the counter/reference electrode is critical to maintain a well-defined electrochemical environment at the working electrode. The biofilm formation was further confirmed by microscopic imaging of the gold electrodes (Figure 1 c) after 32 days of operation, which shows a thick and uniform biofilm located only at the anode (brown color).

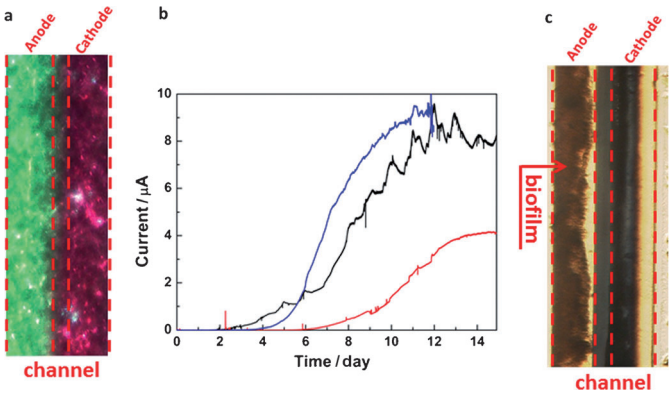

Figure 1. Results of potentiostatically controlled BES: a) microscope image of laminar flow in the microfluidic channel, b) current production by the microfluidic device in potentiostatically controlled mode (three curves with different colors represent triplicate experiments), and c) microscope image of biofilm on the anode after 32 days in brown color. The black color at the cathode was due to the damage of the gold electrode caused by high current densities.

Immediate effects of different chemical stimuli on microbial electrochemical activity of *G. sulfurreducens*

Next, we used our device to study the electrochemical response of *G. sulfurreducens* to certain chemical stimuli. Because *G. sulfurreducens* respires with the electrode by direct electron transfer, electrical current is an excellent indicator of metabolic activity for this microbe. However, most studies focusing on the electrochemical activity of *G. sulfurreducens* have been conducted in bench-scale BESs where i) small changes in mass transfer and velocity distribution can create local disturbances leading to error propagation, and ii) the relatively long HRT (on the order of h vs. min in the microfluidic device) in these reactors makes them unsuitable for testing the effect of short-term shocks on electrochemical activity and prevents rapid screening of stimuli. In our microfluidic device, we create a well-defined environment for the growth of *G. sulfurreducens*, thus, minimizing experimental variability due to subtle changes in the microenvironment and bacterial population. This offers a unique opportunity for in situ monitoring of the phenotypic changes in *G. sulfurreducens* through current production with various chemicals. After the current had reached a stable value in the potentiostatic mode (Figure 1 b), we injected different chemicals into the device in triplicate and recorded changes in the current production. The injection volume and exposure time for all chemicals was 20 μL and 2 min, respectively. The signal with the chemical was statistically compared (2-way ANOVA) to a blank media background [obtained by injecting anaerobic fresh water (FW) media into the device]. In addition, we also ran abiotic stimuli background electrochemical experi-

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ments with all chemicals by injecting them into a sterile device. The abiotic electrochemical contribution to the signal from all the statistically significant chemicals was less than 1% of the signals obtained with the active biofilm, therefore, ensuring that the observed signals can be exclusively attributed to biological activity.

As fumarate is a well-known electron acceptor for *G. sulfurreducens*, injection of fumarate into the device was anticipated to result in a temporary decrease in current because the electrons from microbial metabolism end up in fumarate rather than the electrode.[17] When 140 mM fumarate was injected, the current was reduced by 17.4±0.8% (the decrease was 1.4±0.1 μA, the current before injection was 8.3 μA), thereby confirming the validity of our experimental design (Figure 2a). Recently, there have been multiple studies highlighting the benefits of microaerobic conditions on the operation of BES.[1,18] Lin et al.[19] also demonstrated that *G. sulfurreducens*, which was previously thought to be an obligate anaerobe, could use O_2 as an electron acceptor. Therefore, we chose O_2 as the next chemical of interest. Upon injection of FW medium containing 0.27 mM of O_2, the current was reduced by 3.7±0.1% (decrement was 157±2 nA, the current before injection was 4.2 μA, Figure 2b). However, this decrease was completely reversible as the current recovered back to its original value after O_2 was flushed out of the system. Therefore, an exposure time of 2 min to an O_2-saturated solution is not irreversibly inhibitory to *G. sulfurreducens*.

We chose anthraquinone disulfide (AQDS) as the next chemical stimuli because it has been previously implicated as an electron mediator between *G. sulfurreducens* and iron(III) in soil and we wanted to ascertain whether AQDS (980 μM) can shuttle electrons between bacteria and a solid electrode. The current increased by 28.5±1.6% (the height of the positive peak was 2.1±0.1 μA, the current before injection was 7.5 μA) when we injected AQDS, indicating that this chemical is responsible for long-range electron transfer to the electrode for *G. sulfurreducens* (Figure 2c). To further explore exogenous mediator-based electron transfer by *G. sulfurreducens* with other redox-active compounds, we chose the following chemical stimuli: i) pyrroloquinoline quinone (PQQ, 545 μM) because it is a known redox cofactor in bacteria capable of several redox cycles;[20] ii) riboflavin (2 μM) because *Shewanella oneidensis* MR-1, which is another model electrochemically active organism, is known to use this chemical for electrode-based respiration and it has been recently suggested that riboflavin and cytochrome c can form a redox-active complex;[21] and iii) pyocyanin (PYO, 0.025 μM) because PYO, which is an exocellular electron transfer mediator for *Pseudomonas aeruginosa*, has been shown in BES co-cultures to stimulate current production in a fermenting bacterium.[22] All these three chemical stimuli caused a current decrease (Figure 2d–f). However, compared to the blank media background (−45±0.4 nA for riboflavin; −276±50 nA for PQQ and PYO), the effect of these chemicals was not statistically significant (Figure 3). Thus, these stimuli did not affect the current generation of *G. sulfurreducens*. To eliminate the possibility of abiotic redox reactions of the six stimuli on the gold electrode, abiotic stimuli background experiments were conducted as well. The signals obtained from abiotic stimuli background experiments were significantly smaller than the bacterial current production responses to chemical stimuli
(<1%, Figure 3). Based on the results shown above, we believe that the anthraquinone ring is most efficient at electron transfer with the outer membrane cytochromes of *G. sulfurreducens*. Further in silico molecular dynamic studies would be necessary to substantiate this theory.

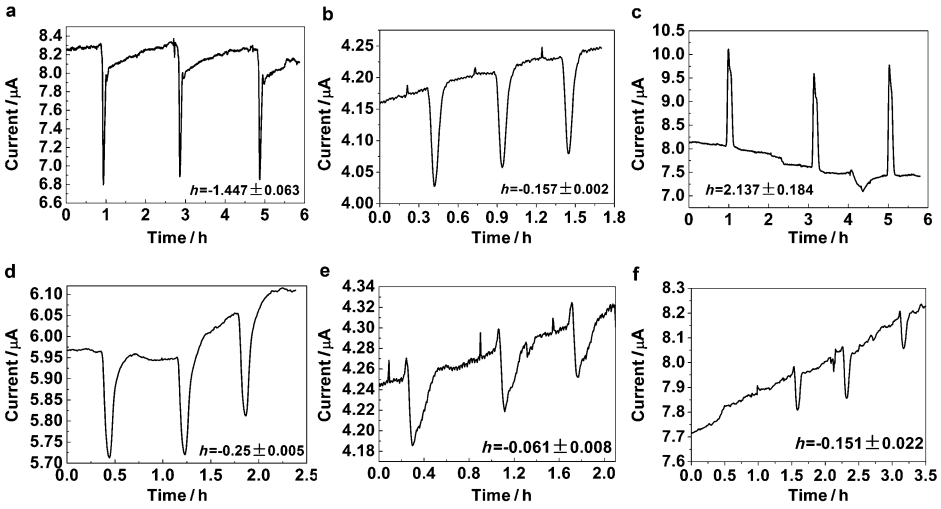

Figure 2. The effect of short term exposure of different chemical stimuli on the electrochemical activity of *G. sulfurreducens* (*h* = height of the peak): a) 140 mM sodium fumarate in FW medium, b) 0.27 mM oxygen in FW medium without cysteine sulfide, c) 980 μM AQDS in FW medium, d) 0.55 mM PQQ in FW medium, e) 2 μM riboflavin in FW medium, and f) 0.02 μM PYO in FW medium.

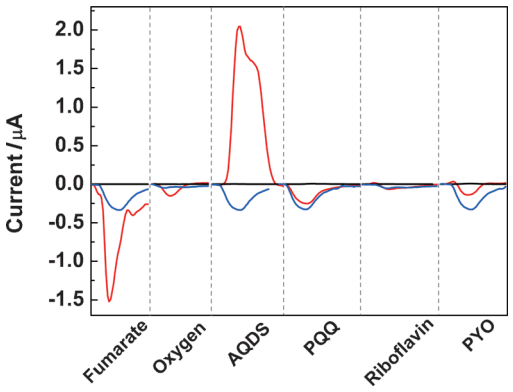

Figure 3. Comparison between bacterial current production response to biologic electric current (——), blank media background (——), and abiotic stimuli background (——).

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Conclusions and Outlook

We tested a microbial bioelectrochemical system (BES) with a laminar flow to separate the two half reactions at each electrode without a membrane. In addition, we demonstrated a possible application of this device: as a research tool for monitoring the effect of various chemical stimuli on electric current production in a potentiostatic mode of operation. We determined that anthraquinone disulfide (AQDS) can serve as a redox mediator for *Geobacter sulfurreducens* (*G. sulfurreducens*), unlike riboflavin, pyocyanin (PYO), and pyrroloquinoline quinone (PQQ). The mechanism of this redox action merits further research. Thus far, microfluidic BESs have been increasingly geared towards use for power production, whereas our device is also extremely valuable as a BES research tool for investigations into electron transfer mechanisms with model electrode-respiring bacteria. As a research platform, this device offers two significant advantages over bench-scale reactors: i) short retention times, which permit high throughput analysis of the effect of stimuli on electrochemical activity and render the device ideal for use as a biosensor and/or biocomputer; and ii) the ability to perform real-time microscopy for correlating biofilm structure, composition, and formation with current production. We also envision that, due to the existence of a very well defined environment as a result of the physical dimensions of the device, it is suitable for conducting experiments to accurately calculate kinetic parameters for bacterial metabolism and electrochemical activity. In addition to the well-defined flow pattern, many other specific advantages compared with conventional bench-scale BESs, for example, predictable velocity profiles and controllable diffusive mixing, make this system a promising tool for other areas in BES research, such as substrate kinetic studies and high throughput environmental stimuli screening for electrochemically active bacteria. We will also further develop this device as a biosensor to screen the acute and/or chronic toxicity of chemicals to anaerobic microbes.

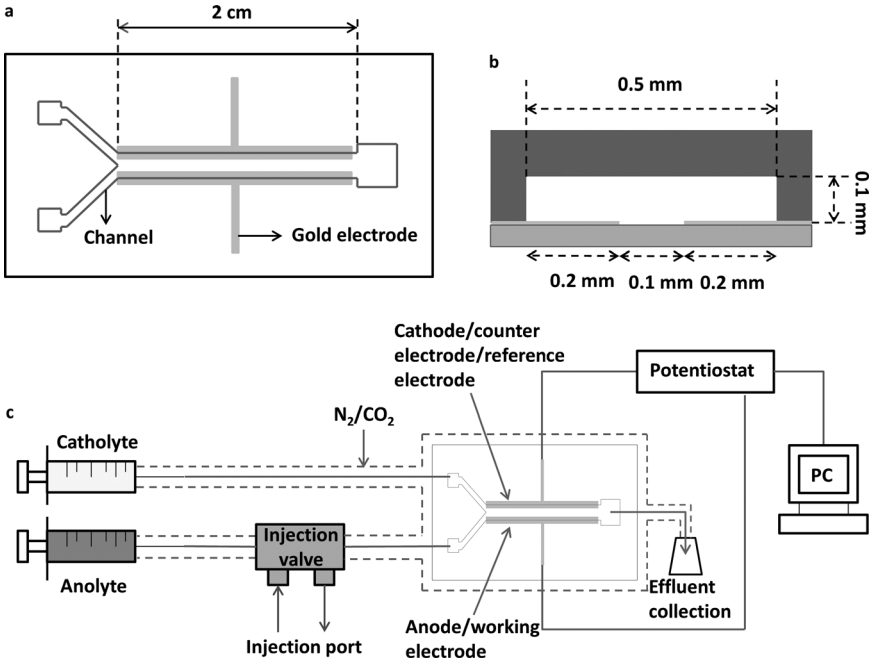
Experimental Section

Bacterial strains and growth conditions

G. sulfurreducens strain PCA (ATCC 51573) was cultured in fresh water (FW) medium containing the following substances per liter of solution: $NaHCO_3$ (2.5 g), NH_4Cl (0.25 g), NaH_2PO_4 (0.52 g), KCl (0.1 g), cysteine sulfide (5 mg), vitamin mix (10 mL; containing 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl·2 H_2O, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg *p*-aminobenzoic acid, and 5 mg lipoic acid per liter), and mineral mix [10 mL; containing 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4$·7 H_2O, 0.5 g $MnSO_4$·H_2O, 1 g NaCl, 0.1 g $FeSO_4$·7 H_2O, 0.18 g $CoSO_4$·7 H_2O, 0.1 g $CaCl_2$·2 H_2O, 0.18 g $ZnSO_4$·7 H_2O, 0.01 g $CuSO_4$·5 H_2O, 0.02 g $KAl(SO_4)_2$·12 H_2O, 0.01 g H_3BO_3, 0.01 g Na_2MoO_4·2 H_2O, 0.025 g NiCl·6 H_2O, and 0.3 mg Na_2SeO_3·5 H_2O per liter]. Sodium acetate (10 mM or 50 mM; depending upon the experiment) was added as the electron donor. The medium was successively flushed with N_2 and 80%:20% N_2/CO_2 (Airgas, NY) to remove O_2, and the pH value was adjusted to 6.8. To keep strictly anaerobic environment in the serum bottle, 0.01% cysteine sulfide was added as the oxygen scavenger. The inoculum was grown in serum bottles with FW medium and 40 mM sodium fumarate as the electron acceptor. For device operation, the anolyte did not contain any alternative electron acceptors (i.e., the electrode was the only electron acceptor), and the catholyte contained FW medium basal salts.

Device fabrication and assembly

The Y-shaped channel was fabricated by means of polydimethyl siloxane (PDMS) via standard soft lithography techniques.[23] Briefly, a silicon mold was prepared by spincoating S1827 positive photoresist on a silicon wafer, followed by: i) exposing the wafer to UV light through a patterned photomask (ABM contact aligner), ii) baking the wafer in a NH_3 image reversal oven, iii) washing the wafer in MIF100 developer, and iv) etching the silicon wafer down to 100 μm thickness by using a Unaxis Si etcher system (SLR 770 etcher Unaxis). A 10:1 mixture of Sylgard 184 (Ellsworth Adhesives, WI) elastomer and curing agent was poured on the silicon mold from the previous step and cured at 90 °C for 2 h, which resulted in the PDMS channel (area = 2 cm × 0.5 mm; height = 100 μm; Scheme 1a and b). Finally, two inlet holes and one outlet hole were punched through the PDMS channel. The gold electrode was prepared by depositing 2 nm gold on a Borofloat wafer with titanium as the adhesion layer (1 nm, CVC SC4500 E-gun evaporation



Scheme 1. Schematic of the microfluidic BES: a) Top view of device, b) cross sectional view of the microfluidic channel, and c) schematic of the entire setup, indicating the anaerobic tubing and chamber.

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system). The fabrication was performed in part at the Cornell Nanoscale Facility. Silver wires were glued to the gold electrodes by using silver epoxy (Electron Microscopy Sciences, PA) for connecting to the ultralow current channel on the potentiostat (Bio-Logic VSP 2000, France). Polymethyl methacrylate (PMMA) plates with screws were used for clamping the channel and the electrode together.

Device operation

All experiments were conducted at room temperature (20–25 °C). In the potentiostatic mode, the device was operated as a two-electrode MEC system (working electrode = anode; counter electrode = reference electrode = cathode). The entire microfluidic device was housed in an acrylic chamber that was constantly maintained under positive 80%:20% N_2/CO_2 overpressure[8] via a brass T-junction connected to the hose barbs. A syringe pump (PHD 2000, Harvard apparatus, MA) equipped with two 100 mL gas-tight gas chromatographic syringes (1100TLL, Hamilton, NV) separately pumped the anolyte and catholyte into the microfluidic device through Tygon microbore PVC tubing (inner diameter = 0.051 cm; outer diameter = 0.152 cm, www.smallparts.com; Scheme 1c). To prevent any oxygen diffusion from the atmosphere into the device, all Tygon tubing was housed in Norprene tubing (06404-18, Cole-Parmer, IL). An injection valve with a 20 μL stainless steel injection loop (HYPO tube 316-RW 16GA, www.smallparts.com) was installed in the anolyte inlet for injecting inoculum, substrate, and chemicals of interest. We used the syringe pump to inject inoculum via the injection valve. When the inoculum reached the gold electrode, the pump was shut down for 2–10 h to allow initial attachment of *G. sulfurreducens* to the anode. Then, operation of the pump was restarted at 1–2 $\mu L\,min^{-1}$ with FW medium containing 50 mM acetate. Once the current started to increase, the flow rate was increased by 1 $\mu L\,min^{-1}$ per day to 10 $\mu L\,min^{-1}$ to maintain a thin and uniform biofilm. Laminar flow and biofilm imaging were performed by using a microscope (KH-7700, HIROX, NJ). Statistical tests were performed with SYSTAT 12 (Chicago, IL). A p-value cutoff of 0.01 (2-way ANOVA) was used to determine statistical significance.

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Keywords: biosensors · electrochemistry · microbes · microbial electrochemical activity · microfluidics

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